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Decellularization of Spinach Leaves as an Outreach Initiative for Introducing Tissue Engineering to Elementary School Students

Clare Flanagan and Kimberly Forsten-Williams, Ph.D.

Abstract

Tissue engineering is a focus area in biomedical engineering with the potential to revolutionize clinical medicine. Removal of cells from existing tissues including plants has emerged as one possible vehicle for the development of new tissues. This project investigates the optimal conditions under which spinach leaves are decellularized using physical (freezing) and chemical treatment (10% Sodium dodecyl-sulfate (10% SDS)). This was done as part of an outreach initiative to create a multilayered scientific protocol accessible to elementary school children to introduce them to tissue engineering. Several variables were tested to determine the ideal conditions that result in optimal decellularization of the leaf, including fresh versus frozen leaves, length of time, kale versus spinach, and several combinations of these. Each experiment compared two petri dishes with one leaf each, one submerged in deionized water and the other submerged in 10% SDS. The best results occur when spinach leaves are frozen for one week, then submerged in 10% SDS for one additional week. This optimization was determined by comparing the color of the spinach from the beginning to the end of the experiment, evidenced by a stark change in color from green to translucent. We conclude that the decellularization experiments culminate in a simple but effective protocol, which includes scientific concepts interwoven with the experiment, and is accessible to elementary school children. The outreach component of the project is accomplished with a virtual presentation accompanied by individual lab kits sent to schools to accommodate a remote teaching experience.

1. Introduction

Tissue engineering is an important branch of biomedical engineering that approaches medical problems through an engineering perspective. The three pillars of tissue engineering are cells with the ability to form a functional matrix, bioreactive molecules such as growth factors that support and induce growth, and finally scaffolds, which provide the other two elements with a stable, transplantable environment upon which to interact and integrate into the host. Scaffolds are the main focus of this project. (**Figure 1.1**)

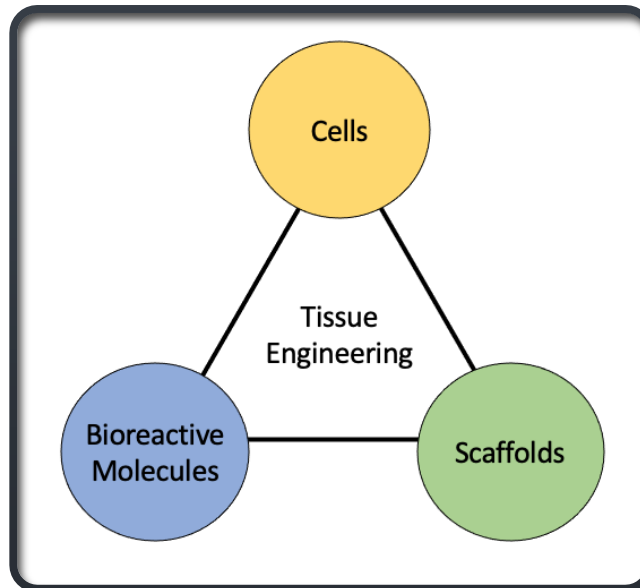


Figure 1.1: The tissue engineering triad composed of bioreactive molecules, cells, and scaffolds.

Like the name implies, scaffolds are an auxiliary structure used to support material in the construction process, not unlike a scaffold for a building. Scaffolds generally need to be biocompatible, which means that the host will not have an adverse reaction to the material when implanted. Scaffolds themselves are fairly easy to synthesize as many materials, both natural and synthetic, meet the biocompatibility criterion; however, in many tissue engineering applications, it is necessary to have a vascular network to supply the tissue with nutrients. Furthermore, synthesizing vessels into an appropriate scaffold structure is rather difficult. Despite being from a different kingdom, plant material is excellent scaffold material since the vasculature is similar to that of animal tissue in that they both branch and taper. We can take advantage of these similarities in tissue engineering since plants have already done the hard work of creating vessels. Our project focused on isolated natural scaffolds through removal of cells rather than building the scaffold from natural or synthetic proteins. In the decellularization process, the native cells are removed through the use of a detergent leaving just the extracellular matrix and vascular network behind. New cells can be added via a process called reseeded (**Figure 1.2**) Spinach was chosen in particular for this project because of its visible pigmentation, large vessels, and low number of tissue layers.

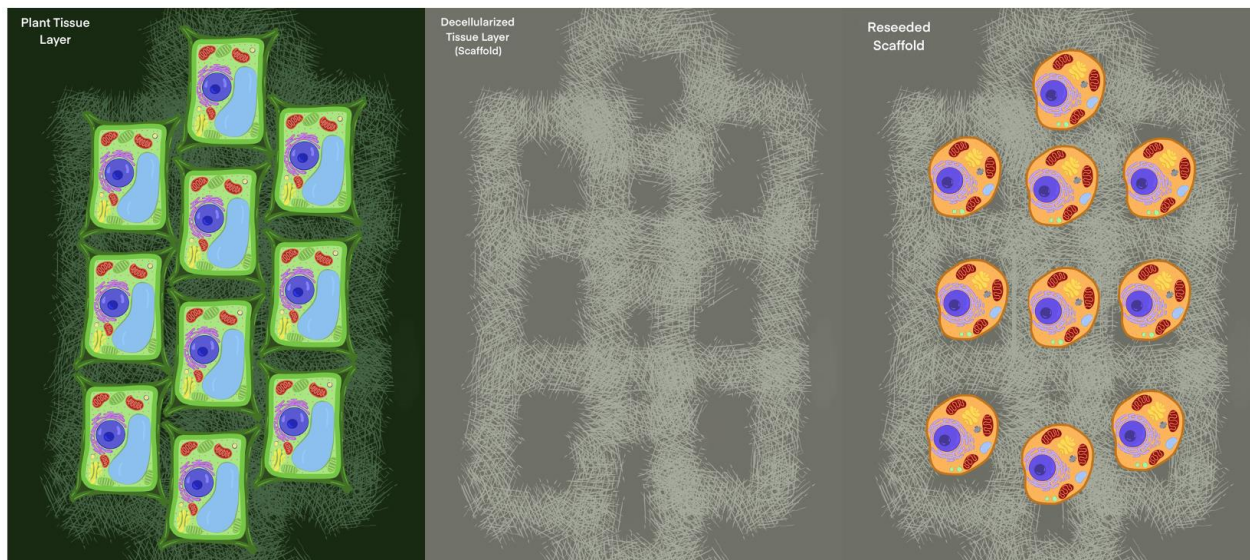


Figure 1.2: Native plant tissue (left) is decellularized through the use of a detergent until all that is left is extracellular matrix (middle) and vascular structures. These remaining structures can then be used to reseed new cells from any cell line, including mammalian cells (right). These new cells, in addition to added bioreactive molecules such as growth factors, are then able to integrate with the host and regenerate tissue layers.

The goal of this project was to develop a simple protocol demonstrating concepts of biology, chemistry, physical science, engineering, and the scientific method while still being safe, manageable, and engaging for students of all ages. The protocol could then be taken into the community along with lab materials and demonstration videos to promote STEM and offer underserved students an engaging scientific experience. Since it was developed during a pandemic, remote instruction was considered an essential part of distributing the project to the community.

2. Materials and Methods

The protocol developed was a simplified version of that used by Gershlak et al (2017). Spinach was obtained from a local grocer and two leaves were sealed flat in a Ziploc bag. They were then placed in a regular freezer for several days. On the day of the experiment, the bags were removed from the freezer and quickly placed flat in two separate petri dishes. In one dish, approximately 25 mL of deionized (DI) water was pipetted over the leaf. The lid was then sealed with masking tape and labelled with “DI water,” initials, and the date. In the other dish, approximately 25 mL of 10% sodium dodecyl-sulfate (10% SDS) was pipetted over the leaf. The lid was then sealed with masking tape and labelled with “10% SDS,” initials, and the date. These dishes were then placed on a counter and left to sit for one week. Their progress was observed after one week, recorded, and left for one additional week. This process was repeated with two more dishes, but these were instead left on an orbital shaker for one week. At the conclusion of the time frames, the scaffolds were compared between experiments and against each of their controls. Ponceau stain was added to DI water in a beaker, and approximately 2 mL was drawn into a syringe. A needle was added to the syringe and inserted into the petiole of the spinach scaffold, which was held gently between two fingers about 5mm away from the petiole (stem side up). Ponceau stain

solution was then injected into the vascular network of the spinach scaffold so that the vascular network could be visibly displayed.

3. Results

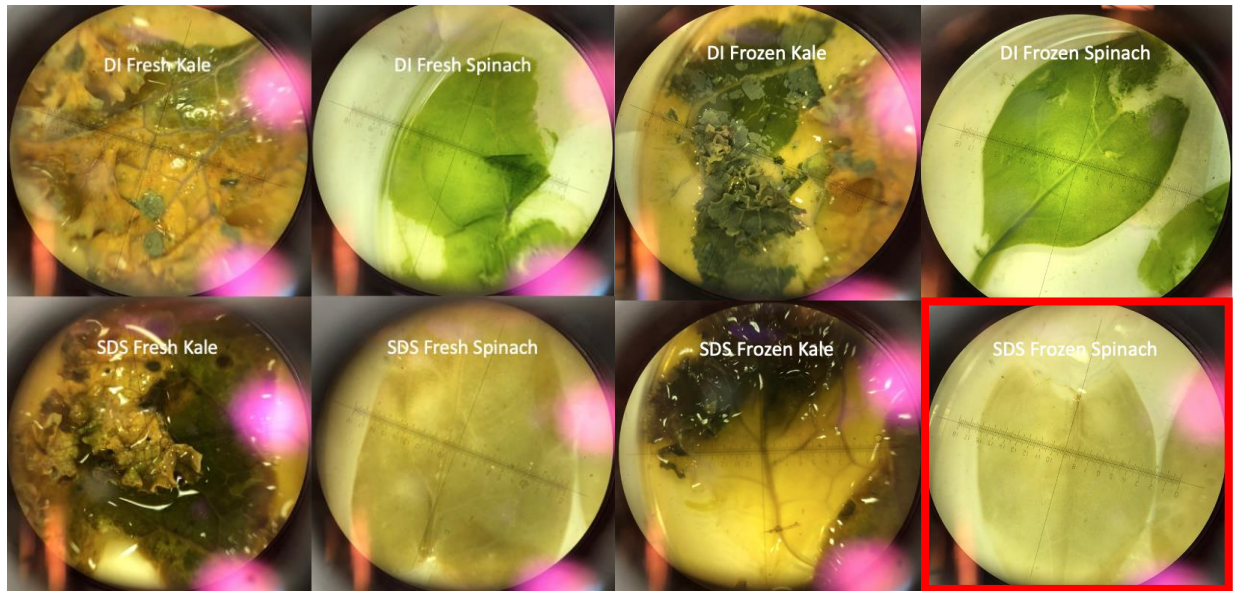


Figure 3.1: Kale and spinach were tested to determine which would display greater translucency and therefore more complete decellularization. Samples were compared both from fresh and from frozen. Frozen spinach proved to be the most translucent after one week had elapsed, so it was selected as the protocol's recommended plant donor.

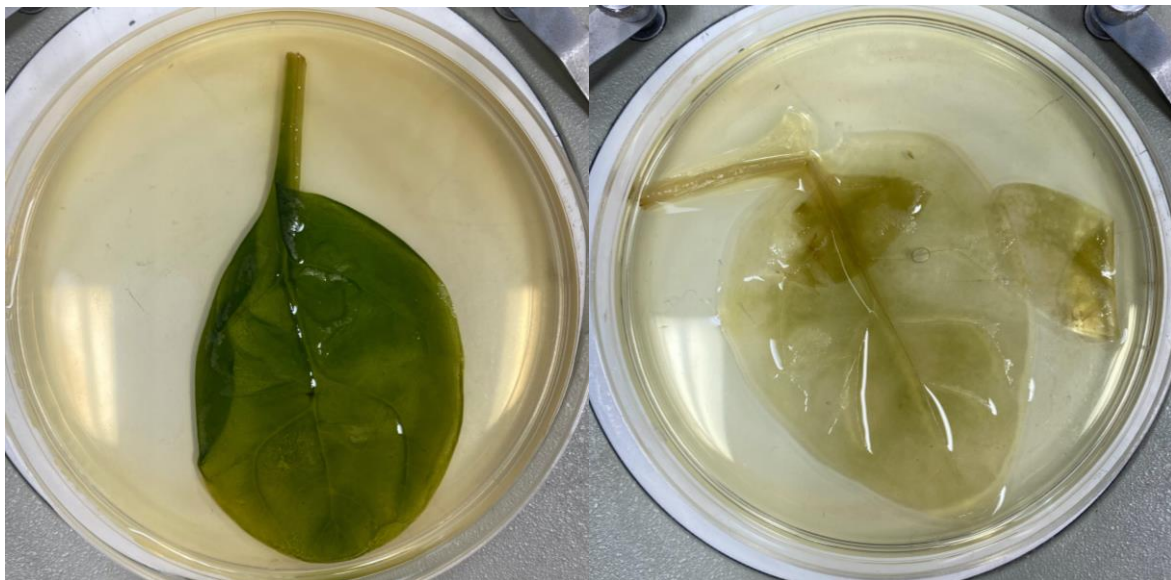


Figure 3.2: The left leaf was statically soaked in DI water for one week while the right leaf was statically soaked in 10% SDS for one week. The SDS has dissolved much of the cellular content, resulting in the leaf's translucency compared to its still-green DI control.



Figure 3.3: The two setups tested are the static setup (left), used for all elementary protocol elements, and the dynamic setup (right), used for college protocol elements, through the use of an orbital shaker to better encourage SDS to react with cells

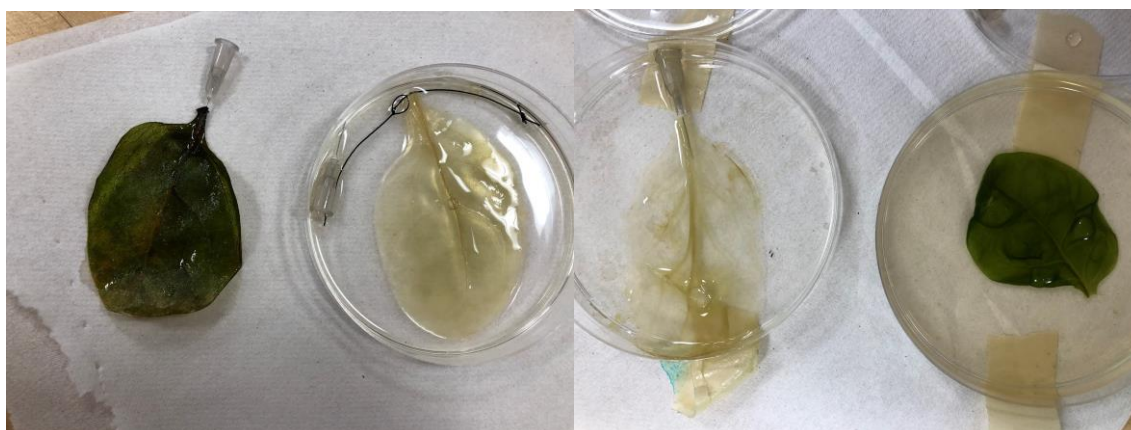


Figure 3.4: Spinach in SDS statically for two weeks (left) reached a degree of translucency similar to that of spinach in SDS dynamically for one week (right).

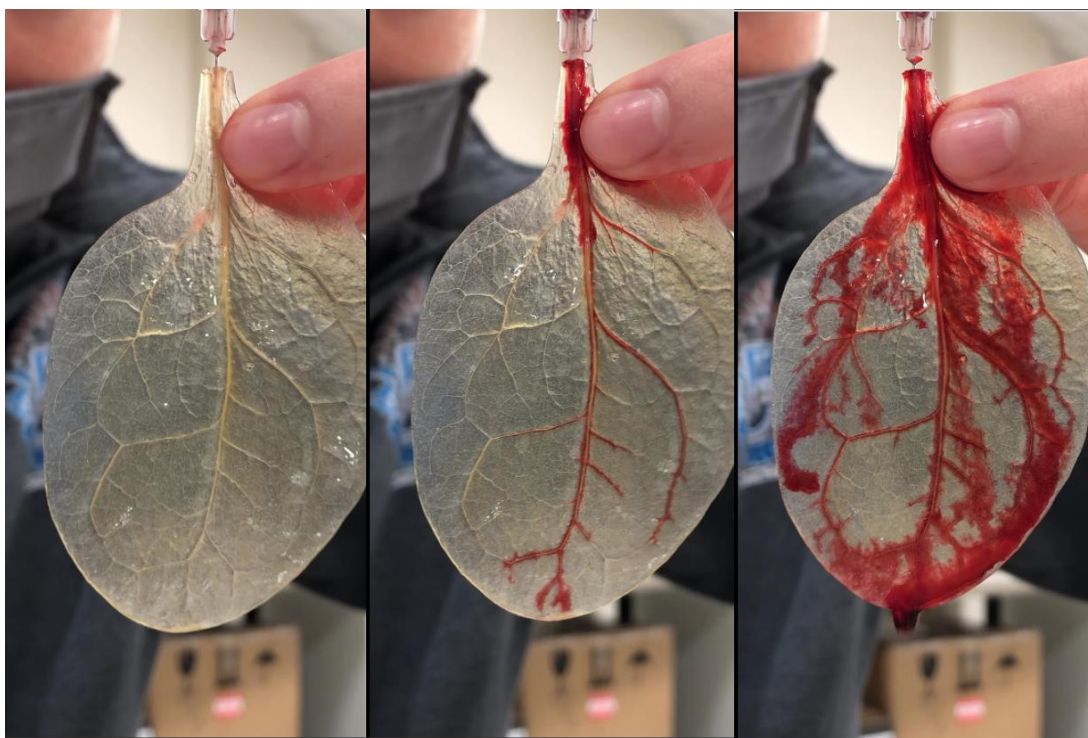


Figure 3.5: Progression of dye perfusion through vascular network of decellularized spinach leaf. Vessels are left intact and are usable by reseeded cells to encourage nutrient transport and faster integration into host tissue.

4. Discussion

The best results are defined as the conditions that yield the most translucent scaffolds over time. Since the pigment chlorophyll contained within the cell's chloroplasts is responsible for color, the leaves become more and more translucent as the SDS combined with the freezing process releases the chloroplasts along with other cellular components. The degree of translucency was taken as an indicator of the effectiveness of the decellularization protocol. It is a simple and low-tech way for elementary school students to visualize the process.

Kale proved to be a poor choice as a scaffold donor in preliminary experiments due to its uneven decellularization and the presence of mold likely due to its irregular anatomy. The kale leaf was not completely submerged in fluid, which likely contributed to the mold growth. In contrast, the spinach reacted very well to the protocol (**Figure 3.1**). The spinach formed no mold in any test that was run, and the increase in transparency occurred evenly since it could lie flatly in the petri dish and be uniformly submerged in the fluid. Spinach was chosen as our preferred scaffold donor with a two-step process of freezing and SDS incubation (**Figure 3.2**).

A more advanced protocol geared toward a college audience was created as well. It uses frozen spinach in its materials and methods just as the elementary protocol does but added a dynamic component in addition to the static setup. In the dynamic version, the setup was placed on an orbital shaker (**Figure 3.3**) for one week. Agitation was thought to improve rate of decellularization by mechanically inducing fluid mixing and removal of cell components. The rate of decellularization of the dynamic experiment could then be compared to that of the static experiment. To the naked eye, two weeks of static culture was approximately equal to one week

of dynamic culture in terms of relative translucency (**Figure 3.4**), but the college class may have different findings through repeated trials. Additionally, they would be able to use perfusion via a ponceau stain solution to show the vessels at the conclusion of decellularization (**Figure 3.5**).

5. Conclusion

The scaffolds produced by this experiment are potentially viable for reseeding with new cells such as from a mammalian cell line, and this reseeding component is one avenue of a future iteration for the college level protocol. Additionally, growth factors could also be added in order to complete the tissue engineering triad and produce a viable implant. For the college protocol, mathematical modeling and protein/DNA mapping aspects are potential future directions. Further applications of plant scaffolds used in medicine are detailed in the paper by Gershlak et al (2017) where they seeded their spinach decellularized scaffold with cardiomyocytes, which actually began to spontaneously contract about 7 days after seeding on the plant tissue scaffold. The scaffolds produced by this project need to be studied quantitatively before reseeding can occur, but they show promise for future studies.

The plan for the outreach component was to package lab kits with all the necessary materials and take them to three schools with various socioeconomic statuses and therefore different science budgets and create an interactive demonstration video. The materials created and developed for this include detailed presentations, prepared lab kits, a demonstration video publicly available, and an accompanying worksheet for the elementary protocol. The worksheet adequately explains the lab and highlights all the concepts it encapsulates, which are biology, chemistry, physical science, engineering, and the scientific method. The goal of this lab is to present all of these concepts in an engaging way while simultaneously promoting STEM. All these materials for taking this into the community are ready and available for future use. However, despite its remote-teaching features, outreach activities were tabled due to pandemic-related school closures.

6. Acknowledgements

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